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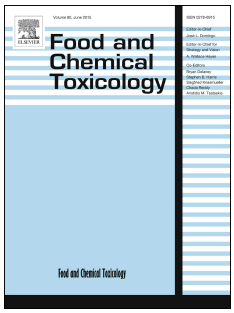

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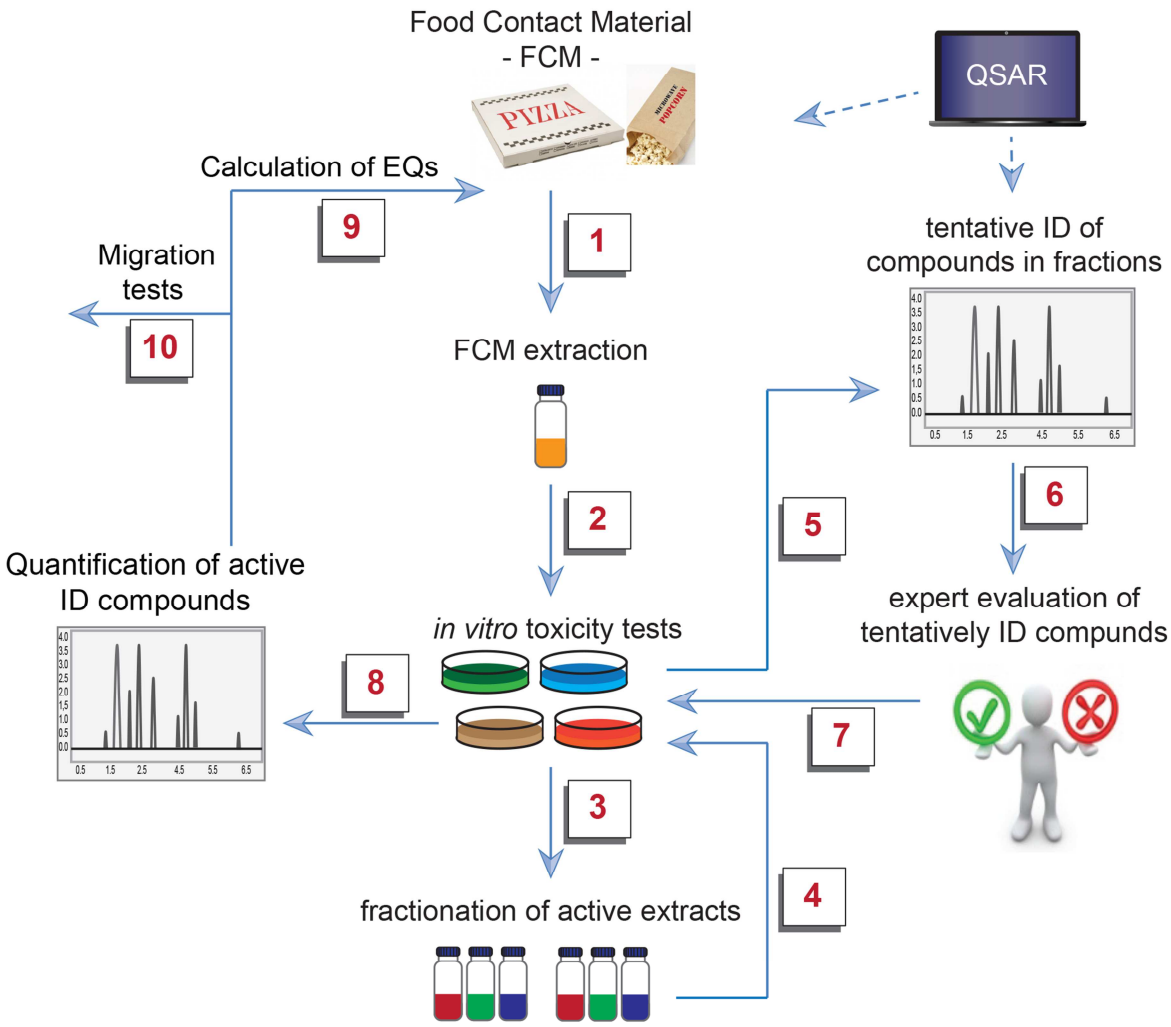
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Running Title

Hazard identification strategy for food packaging

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Abstract

Food contact materials (FCM) are any type of item intended to come into contact with foods and thus represent a potential source for human exposure to chemicals. Regarding FCMs made of paper and board, information pertaining to their chemical constituents and the potential impacts on human health remains scarce, which hampers safety evaluation. We describe an effect-directed strategy to identify and characterize emerging chemicals in paper and board FCMs. Twenty FCMs were tested in eight reporter gene assays, including assays for the AR, ER, AhR, PPARγ, Nrf2 and p53, as well as mutagenicity. All FCMs exhibited activities in at least one assay. As proof-of-principle, FCM samples obtained from a sandwich wrapper and a pizza box were carried through a complete step-by-step multi-tiered approach. The pizza box exhibited ER activity, likely caused by the presence of bisphenol A, dibutyl phthalate, and benzylbutyl phthalate. The sandwich wrapper exhibited AR antagonism, likely caused by abietic acid and dehydroabietic acid. Migration studies confirmed that the active chemicals can transfer from FCMs to food simulants. In conclusion, we report an effect-directed strategy that can identify hazards posed by FCMs made from paper and board, including the identification of the chemical(s) responsible for the observed activity.

1. Introduction

Food contact materials (FCMs) are materials intended to come into contact with foods, from processing equipment through to kitchen appliances and packaging. FCMs thus constitute a vast collection of products that individually can contain a large number of chemicals (Muncke et al., 2014). Humans can be exposed to these chemicals if they migrate to the food (Borchers et al., 2010), which ultimately may contribute towards causing adverse health effects. Since data pertaining to both occurrence and toxicity of a large number of chemicals that can be present in FCMs are limited, it remains difficult to assess what potential risks they may pose to human health. Among the many types of FCMs, those made from paper and board are particularly interesting in this regards, as there are still no specific EU regulations in place for these. Notably, the EU framework regulation from 2011 and 2016 do cover FCMs more broadly, stating that compounds should not transfer from FCMs into food in amounts that can adversely affect human health (EU, 2011, 2016). But since this does not adequately address specific chemical constituents, novel strategies to identify potential hazards from FCMs are needed. This means that more occurrence data needs to be collected alongside robust testing strategies designed to evaluate biological activities of the materials themselves as well as identified compounds therein.

FCMs made from paper and board can contain chemicals that have been either added intentionally as active ingredients, or that occur unintentionally as byproducts, impurities, or degradation products. Compounds may also originate from cellulose-based materials or be introduced through the recycling process. Examples of substances detected in FCMs of paper and board are polyfluoroalkyl substances (Schaider et al., 2017), bisphenol A, phthalates (Lopez-Espinosa et al., 2007a), mineral oil hydrocarbons (Lorenzini et al., 2010), and heavy metals (Conti, 1997). Some of these are suspected to cause adverse effects, for instance

bisphenol A at low doses can affect anogenital distance (Christiansen et al., 2014), disturbe mammary gland development (Moral et al., 2008) or behavior in offspring (Xu et al., 2010). Further, some polyfluoroalkyl substances have been reported to cause hepatomegaly, tumor induction in liver, pancreas or testis, developmental effects, and immunotoxicity (Lau, 2012). Collectively, this exemplifies that FCMs of paper and board can be chemically very complex and may contain substances with known adverse effects.

Employing classical approaches such as targeted analysis to characterize the chemical composition of the FCMs and successively testing single compounds for biological activities is therefore inadequate, as it will neither provide any information for compounds that are not explicitly known to be present in the material, nor account for the total, integrated biological activity of all the compounds present in the product– 'the cocktail effect'. To address these shortcomings, an effect-directed strategy could be applied, as exemplified in previous studies by us and others. However, although these earlier strategies were based on *in vitro* tests for genotoxicity, cell toxicity, or endocrine activity, in combination with advanced analytical chemistry to identify the active compounds in FCMs (Binderup et al., 2002; Lopez-Espinosa et al., 2007b; Ozaki et al., 2004; Vinggaard et al., 2000; Weber et al., 2006), they only included a few *in vitro* endpoints or a small amount of FCM samples, or failed to fully identify the causative compounds. Thus, an improved strategy is needed to obtain good and broad toxicity profiles, as well as enhancing the identification process.

To enhance existing testing procedures of FCMs made from paper and board, we aimed to develop an effect-directed strategy that combines a broad panel of *in vitro* assays with state-of-the art analytical chemistry. This was done to better facilitate the identification of potential problematic paper and board FCMs, but focused specifically on improving the identification of potentially hazardous compounds. As a proof-of-principle, twenty FCMs of paper and

board were partly analyzed by the effect-directed analysis to identify biological activities, of which two FCMs were subjected to the entire step-by-step procedure attempting to identify biologically active constituents.

2. Materials and methods

2.1 Strategy work-flow

The strategy for FCMs of paper and board includes ten steps from extract preparation to identification of compounds with biological activity and determination of migration of these (Figure 1).

2.2. Test compounds and chemicals

Chemicals used for producing extracts and fractions are described elsewhere (Bengtstrom et al., 2014). All aqueous solutions were prepared using ultrapure water obtained from a Millipore Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). HPLC-MS grade formic acid and a water solution of 25% ammonium hydroxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC TOF-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Standards for the LC-qTOF method: Di-n-butyl phthalate (DBP) (CAS: 84-74-2) (99%), deuterated di-n-butyl phthalate (d_4-DBP) (CAS: 93952-11-5) (>98%), benzyl-butyl phthalate (BBP) (CAS: 85-68-7) (99%), di-isobutyl phthalate (DiBP) (CAS: 84-69-5) (99%), bisphenol A (BPA) (CAS: 80-05-7) (99%), methylparaben (CAS: 99-76-3) (99%), bisphenol A diglycidyl ether (BADGE) (CAS: 1675-54-3) (95%), perfluorooctanoic acid (PFOA) (CAS: 335-67-1) (95%), abietic acid (AA) (CAS: 514-10-3) (75%), dehydroabietic acid (DHAA) (CAS: 1740-19-8) (95%), isorhamnetin (CAS: 480-19-3) (99%) and rhamnetin (CAS: 90-19-7) (99%) were all obtained from Sigma-Aldrich and 4-oxo-retinoic acid (CAS: 150737-18-1) (98%) were obtained from Santa Cruz Biotechnology, TX, USA. Stock solutions for *in vitro* testing of DBP, BBP, DiBP, BPA, AA, DHAA, isorhamnetin, rhamnetin, and 4-oxo-retinoic acid were prepared in DMSO at 40-50 mM.

2.3 Quantitative structure–activity relationship (QSAR) screening of FCM compounds

A QSAR screen was performed for 2,076 known FCM compounds. Initially, a consolidated list of 4,041 unique compounds – including additives, monomers, solvents, photo-initiators, dyes, and pigments – was compiled using two publicly available sources: (Council_of_Europe, 2009) and (Federal_Office_of_Public_Health, 2011). Of these, in-house structural information was available for 2,076 compounds; the final number included in the QSAR screen consisting of a combination of models for genotoxic carcinogenicity, mutagenicity, developmental toxicity, and endocrine activity. Detailed information on the performance of the individual models, the applied decision algorithms, and the method for preparation of the structure set have been described previously (Wedebye et al., 2015). According to validation results, the applied models have prediction accuracies of 70-85%.

2.4 FCM sample selection and production of extracts

Twenty paper and board FCM samples were obtained from retailers or manufacturers (Table 1). The selection criteria were a) consideration regarding starting material of the FCM (i.e. virgin *vs* recycled), b) the presence of printing inks, c) the intended conditions of use, and d) the type of food used in contact with the material.

The FCM extracts and fractions were prepared as previously described (Bengtstrom et al., 2014). Briefly, double-sided extraction of the FCMs (37-112 dm^2) was performed in 650 mL ethanol for 4 h under reflux, before successively evaporated to an average concentration of 32.8 ± 9.8 dm^2/ml. The two FCM extracts S3 and S7 were subjected to the entire strategy, starting with fractionation by HPLC under both alkaline and acidified eluent conditions. Reproducibility of the extraction method has been published previously (Bengtström et al. 2014).

2.5 In vitro testing of extracts, fractions, and identified compounds

In vitro tests were performed using eight reporter gene assays: Androgen receptor (AR), Estrogen receptor (ER), Aryl hydrocarbon receptor (AhR), Peroxisome proliferator-activated receptor γ (PPARγ), Glucocorticoid receptor (GR CALUX), Retinoic acid receptor (RAR CALUX), Nuclear factor (erythroid-derived 2)-like 2 (Nrf2 CALUX), and Transformation-related protein 53 (p53 CALUX), essentially as described previously (Piersma et al., 2013; Rosenmai et al., 2014; Rosenmai et al., 2016; Taxvig et al., 2012; Van der Linden et al., 2008; Vinggaard et al., 2002). All assays were run in agonist mode, however the AR assay was also run in antagonist mode (0.1 nM R1881 added). To validate assay performance, positive control compounds were included: rosiglitazone for PPARγ assay (1 µM); 2,3,7,8-tetrachlorodibenzo-p-dioxin for AhR assay (0.5E-12 to 3E-9 M); 17β-estradiol for ER assay (0.36E-12 to 367E-12 M); R1881 (agonist)(1.2E-12 to 2.7E-9 M) and hydroxyflutamide (antagonist) (1E-9 to 5E-6 M) for AR assay; all-trans-retinoic acid for RAR CALUX assay (15E-12 to 50E-6 M); actinomycin D for p53 CALUX assay (0.15E-12 to 0.5E-12 M); dexamethasone for GR CALUX assay (15E-15 to 50E-9 M); curcumine for Nrf2 CALUX assay (5E-12 to 17E-6 M).

FCM extracts were tested at a maximum concentration of 0.25-1.0% of original extract over ten dilutions varying in fold-dilutions between 2 to 3.3 dependent of assay. Experiments were performed using 3-6 replicates and repeated in 1-3 independent experiments. The S3 and S7 fractions were tested for AR and ER activity, respectively, at 0.25% of the original fraction. The tentatively identified FCM compounds DHAA, AA, rhamnetin, isorhamnetin, and 4-oxo-retinoic acid (in the AR assay), as well as DiBP, BBP, and DBP (in the ER assay) were all tested using concentrations ≤100 µM across 2-3 experiments performed with 3-4 replicates. Ames test for genotoxicity was performed using five concentrations in triplicate

reactions and repeated twice following protocols as previously described (Binderup et al., 2002).

2.6 Identification of FCM compounds

For the tentative identification process, extracts and corresponding fractions of selected samples (S3 and S7) that exhibited *in vitro* activity were analyzed by GC-QTOF or UPLC-QTOF (Agilent Technologies, Santa Clara, CA, USA), in 1:100 v/v dilutions. For GC-QTOF, a standard mixture (10, 100 and 500 ng/mL of DBP, d_4-DBP, BBP, DiBP) was analyzed before and after all test samples, and electron ionization performed at 70 eV. For LC-ESI-QTOF the standard mixtures consisted of 100 and 500 ng/mL of BPA, methylparaben, BADGE, PFOA and AA. For GC-QTOF, data analyses were performed with the Agilent MassHunter Qualitative software (NIST library v.11), whereas LC-ESI-QTOF data analyses were performed with the ProGenesis QI software (Nonlinear Dynamics Limited, UK) in both positive and negative ionization mode.

To streamline the tentative identification process, threshold of interest for peaks was based on Thresholds of Toxicological Concern (TTC) for compounds with suspected genotoxic effects (EFSA Scientific Committee, 2012). A TTC of 0.15 µg/person/day was used to calculate a threshold of interest of 125 ng/6 dm^2, and assuming an intake of 1 kg food/person/day packed in 6 dm^2 of FCM. The cut-off level was set at 12.5 ng/dm^2 for d_4-DBP analyzed by GC-QTOF, 1.25 ng/dm^2 for BADGE analyzed by LC-QTOF in positive ESI mode, and 1.25 ng/dm^2 for PFOA in LC-QTOF in negative ESI to account for differences in detector response and ion suppression. Peaks with areas below this threshold were excluded. Tentative identification of LC-ESI-QTOF data was performed using a customized library containing

approximately 2,300 matrix-specific entries (Bengtström et al., 2016), and the ChemSpider and PubChem database.

Selected tentatively identified compounds were confirmed and quantified by chemical target-analysis. Phthalates were quantified in 1:1000 v/v ethanol-diluted samples using a GC-MS method (Petersen and Jensen, 2010) with minor modifications. Briefly, internal d_4-labelled standards were added to a sub-sample of food simulant, which was further diluted with water before the phthalates were liquid-liquid extracted using cyclohexane. Phthalates were separated on a 30 m non-polar DB-5 capillary column and detected in the single ion monitoring mode, using one ion for quantification and two ions to verify identity. GC-QTOF settings and *m/z* of the quantification and verification ions are described in (Bengtström et al., 2016).

BPA, AA, DHAA, 4-oxo-retinoic acid, isorhamnetin, and rhamnetin were all quantified in 1:1000 v/v ethanol-diluted samples by LC-MS/MS. The method for BPA was based on an accredited HPLC QqQ mass spectrometry (HPLC-MS/MS) protocol as described in Table S1. An eight-point calibration curve of BPA in a methanol/water solution (75:25 % v/v) was used (0, 7.5, 15, 30, 75, 150, 225 and 300 ng/mL). The internal standard d_{16}-BPA (150 ng/mL) was added to both calibration standards and extracts. The mass transition reactions used for BPA quantification were *m/z* 227.2 > 212.1 and, *m/z* 227.2 > 133.1 as qualifier and *m/z* 241.2 > 223.1 for d_{16}-BPA. For the remaining compounds, a seven point calibration curve of a standard mixture of AA, DHAA, 4-oxo-retinoic acid, isorhamnetin, and rhamnetin in ethanol (0, 10, 20, 50, 100, 200, and 500 ng/mL) was used. Masses used for quantification of AA were *m/z* 301.5 > 301.5 and for DHAA *m/z* 299.5 > 299.5. The calibration curves for

all methods had linearity of $R^2 > 0.98$. The method is described in Table S1. Data were analyzed by the Waters QuanLynx (v 4.1) software.

2.7 Data processing, statistical analyses and EQ calculations

The criteria for determining if an extract displayed *in vitro* activity were i) that the mean values between treatment groups exhibited a statistically significant difference ($p < 0.05$), ii) the effect was dose-dependent, and iii) the effect was observed in the majority of the independent experiments. For the PPARγ, Nrf2, RAR, GR, and p53 reporter gene assays, tentative Lowest Observable Effect Concentrations (LOECs) were found for each FCM. For AR antagonism data, LOECs were determined as the concentration at which ≥ 25% inhibition was observed. For AR, AhR, and ER agonism data, LOECs were determined when ≥ 50% increase in response was observed. The maximum response change was calculated as the difference between control and maximum induction/inhibition in percentage for all extracts.

A four-parameter sigmoidal curve fit was used for *in vitro* data obtained from the S3 and S7 extracts. The limits of the model were fixed at 1 and the maximum fold-induction/inhibition. The same model was used for the identified compounds and positive controls. Estimated Hill-slopes and EC_{50} values were used to determine estrogen equivalence factors (EEQs) and anti-androgen equivalence factors (AEQs) for extracts (EQ_{meas}) and for compounds identified in fractions and corresponding extracts (EQ_{calc}). The following equations were used to calculate EQs:

(1) $$response = bottom + \frac{top - bottom}{1 + 10^{(\log(EC_{50}) - \log(concentration)) * hillslope}}$$

(2) $$log(concentration) = log(EC_{50}) - \frac{log\left(\frac{top - response}{response - bottom}\right)}{hillslope}$$

Identified compounds were quantified in the FCM extracts. Based on the parameters obtained from concentration-response curves of identified compounds, the predicted response in the extract was calculated by using equation (1). The inserted compound concentrations were those present in the extract at the maximum fold induction. This calculated response was successively inserted into equation (2), in which all parameters were based on the positive control. By doing so, the concentration of identified compounds was converted into EQs of the positive control. The EQs for individual compounds were summed to obtain the total EQ_{calc}. For the extract, the EQ_{meas} was calculated by inserting the maximum fold induction for the extract into equation (2) with all the parameters in the equation being based on the positive control.

Statistical analysis on data obtained from extracts and tentatively identified compounds was carried out after normalization to vehicle controls. Normally distributed data were analyzed by one-way ANOVA (post-test Dunnett) or alternatively by Kruskal Wallis test (post-test Dunn). GraphPad Prism 5 was used for statistical analyses and mathematical modelling.

2.8 Migration tests

Migration tests were conducted in triplicate with the dry food simulants (Tenax®) or the simulant for e.g. open sandwiches with cheese, egg or cold meat (simulant D2: 50% ethanol) based on intended use of the FCM. Briefly, a 0.5 dm^2 circular piece from the pizza box (S7) was placed in a metal screw cap without gasket. Tenax® (1.77 g) was placed in a 200 mL glass jar which was closed with the metal screw cap. The jar was turned upside down and placed in a climate cabinet controlled for temperature and humidity. The jar was not

completely air tight, so that water content of the paperboard quickly came into a state of equilibrium with the relative humidity (RH) of the cabinet. The pizza box (S7) was tested for 2 h at 70°C at 80% RH with Tenax® simulating dry foods (the bread part). According to EC regulation 10/2011, (Annex 3, table 2, food type 02.05), simulant D2, 50% ethanol in water, is the appropriate simulant for the topping part containing fatty foods (applying a reduction factor of 3). However, since test with simulant D2 cannot reliably be applied at 70ºC it was decided to use the extraction result with 95% ethanol as guidance value. The sandwich paper (S3) was tested for 24 h at 40 °C and 60 % RH% with Tenax® (simulating dry foods) and by total immersion in 50 % ethanol (simulating open sandwiches with cheese, egg or cold meat) according to CEN 13130, part 1. Only compounds with confirmed identity in fractions and extracts as well as biological activity were identified and quantified in migrates by methods described in section 2.6.

3. Results

3.1 QSAR predictions of inventoried FCM compounds

To gain some initial insight into what extent chemical components in FCMs can cause adverse effects, a QSAR screen was carried out for 2,076 compounds reported by manufacturers to be used in FCMs. The results represent positive predictions. No distinction was made between a negative and an unreliable prediction; that is, a prediction outside the applicability domain of the model was simply discarded. A total of 599 compounds, corresponding to 29% of the screened compounds, showed positive predictions for one or more of the endpoints. As depicted in Figure 2, the positive predictions across the six chosen endpoints were: 10% for genotoxic carcinogenicity, 14% for *in vivo* mutagenicity, 9% for developmental toxicity, 4% for AR antagonism, 2% for ER activation, and 3% for ER binding.

3.2 In vitro activities of extracts and fractions from FCMs made from paper and board

Results for positive controls from the different assays are shown in Supplementary Materials. Graphed data represents 3-5 experiments and generally showed good reproducibility between experiments. The dynamic ranges of the assays are indicated by the response to positive controls: TCDD (AhR activator) caused a maximum response change of ~1400%, E2 (ER activator) caused ~400% induction, R1881 (AR activator) caused ~3000% induction, OHF (AR inhibitor) caused ~75% reduction, rosiglitazone (PPARγ activator) caused ~3000% induction, curcumin (Nrf2 activator) caused ~4000% induction, and actinomycin D (p53 activator) caused ~2500% induction.

All twenty paper and board FCMs showed activity in at least one of the eight *in vitro* assays used herein, with the majority being active in multiple tests. These data are summarized in Table 2, with graphs available in Supplementary Materials. Eleven FCM extracts tested positive for AR activity, all for AhR activation, nine for ER activity, twelve for PPARγ activity, sixteen for Nrf2 signal transduction, and six for p53 activity. None of the FCM extracts caused significant effects on RAR or GR activation (data not shown). The microwave pizza tray (S8) and the popcorn bag (S10) extracts were also positive in the Ames test (data not shown).

The potencies and maximum response changes of the FCM extracts varied from 0.002-22 cm^2 FCM/mL and 73-1069% on AhR activity, 0.1-19 cm^2 FCM/mL and 223-1645% on Nrf2 activity, 0.2-6.5 cm^2 FCM/mL and 60-470% on PPARγ activity, 0.4-18 cm^2 FCM/mL and 47-365% on p53 activity, and 0.1-5.9 cm^2 FCM/mL and 63-245% on ER activity. FCM extract-potencies and maximum response changes in the AR antagonist assay were 0.1-22 cm^2 FCM/mL and 28-66%, whereas agonism was observed with potencies of 0.4-11 cm^2

FCM/mL and maximum response changes of 73-532%. Notably, four extracts exhibited both AR agonism and antagonism.

The most pronounced AhR activities were observed for the pizza box (S7) and the tomato punnet (S15). The cereal box (S12), the sausage tray (S14), the tomato punnet (S15), the paperboard with water-soluble print (S19), and the paperboard with offset print (S20) displayed the most significant Nrf2 activity. The cake tray (S13) and the tomato punnet (S15) were amongst the most PPARγ active and the paperboard with UV print (S18) displayed pronounced p53 activity.

The sandwich wrapper extract (S3; made from virgin paper) was selected for the full strategy (Figure 1) due to its marked (anti)androgenic activity, whereas the pizza box extract (S7; made from recycled paper) was selected because of its marked ER activity combined with a marked AhR activity. The concentration-response relationships for these FCM extracts on AR and ER activity, respectively, are shown in Figure 3. The HPLC fractions 8 and 9 of the sandwich wrapper showed marked AR antagonism, whereas fractions 6 and 7 of the pizza box exhibited ER activity (Figure 3).

3.3 Identification of active compounds in FCMs

We tentatively identified 16 and 47 compounds by analytical chemistry in fractions of S3 and S7, respectively. From these, a subset of compounds were selected based on prior knowledge on ligand and biophore interactions with the specific receptors (Jensen et al., 2011), known reported activities, plausible usage, and commercial availability of standards. The final list included BPA, DBP, DiBP, and BBP from the S7 fractions, and DHAA, AA, isorhamnetin, rhamnetin, and 4-oxo-retinoic acid from the S3 fractions. These tentatively identified

compounds were subjected to further *in vitro* analyses. BPA, BBP, and DBP proved to activate the ER receptor (Figure 3), whereas DiBP showed no effect (data not shown). Isorhamnetin, 4-oxo-retinoic acid, AA, as well as DHAA antagonized AR activity (Figure 3), whereof only AA and DHAA were confirmed present in the extract. Rhamnetin was markedly cytotoxic.

Retention times and fragmentation patterns for BPA and the analyzed phthalate standards confirmed our tentative identification from both the ER active extract and the fractions. DHAA and AA – the only AR compounds that had an entry in the customized database – were verified by LC-MS/MS from both extract and fraction. Concentrations at maximum fold change of the identified compounds are listed in Table 3. Our calculations showed that the EEQ_{calc} based on BPA, DBP, and BBP was ~5-fold higher than the EEQ_{meas} in the pizza box (S7), whereas the AEQ_{calc} based on DHAA and AA was 1.7-fold higher than the AEQ_{meas} in the sandwich wrapper (S3) (Table 3).

3.4 Migration of FCM constituents to food simulants

As a final test, the ability of identified chemicals to migrate from FCM into foodstuff was assessed by food-simulant migration assays. Table 4 summarizes data for the original ethanol extraction of samples, as well as the migration tests to 50% ethanol and dry food simulant. The highest transfer rates were seen for AA and DBP. For AA present in the sandwich wrapper (S3), 8% migrated into the food simulants representing cheese, egg and cold meat. For DBP present in the pizza box (S7), 11% migrated by the headspace into the food simulant representing dry foods such as breads. Notably, migration of BPA, DBP, and BBP from the pizza box was only tested with the dry food simulant as the area of "wet" contact between paperboard and pizza-topping is limited and cannot reliably be estimated. Migration was in

all cases lower than the specific migration limits (SML) for FCM made of plastics. Only in the case where full transfer of all DBP that is present in the FCM occurs, would a slight violation of the SML be expected.

4. Discussion

FCMs encompass a diverse group of materials and products that can comprise a complex mixture of ingredients and chemicals. If any of these chemicals migrate to the foodstuff, the consumers can inadvertently be exposed by handling or eating the products. In turn, this can put the consumers at increased risk should they be exposed to the same chemical from other sources or be exposed to other chemicals (from FCM or other sources) that exhibit similar bioactivity. In this study, we have developed an effect-directed strategy to measure the toxicological activity of FCMs and to identify the active chemical constituents.

QSAR predictions were initially carried out to screen around 2,000 compounds that are inventoried for use in FCMs. Although not a required component of the strategy as a whole, it was done to better understand whether FCM constituents can affect defined 'adverse effect endpoints' irrespective of actual real-life exposure levels. Surprisingly, nearly 30% of the ~2,000 compounds were predicted to be positive for at least one of the chosen endpoints, which included genotoxic carcinogenicity, mutagenicity, developmental toxicity, and endocrine activity. Compounds showing alerts for one or more toxicological endpoints could then be assigned a high priority for further evaluation including the potential to migrate and become bioavailable. In addition, biophores identified as affecting specific receptors or activities by QSAR modeling proved valuable for selecting putatively active compounds from those tentatively identified in the FCM extracts. In this study, we used biophores that are known AR antagonists (Jensen et al., 2011). This way we tentatively identified some compounds that could be responsible for the observed AR activity of the extract, later confirmed *in vitro*. Thus, we envision that QSAR predictions will gradually become more integrated into future strategies.

Based on our QSAR predictions and previous experience, we designed a test panel of assays targeting specific endpoints related to endocrine activities, oxidative stress, genotoxicity, and mutagenicity. Assays covering similar steps in toxicity pathways have previously been used for water samples (Escher et al., 2014). By following our strategy, we found that twenty out of twenty tested FCM extracts displayed activity in at least one assay. Although we did not identify the causative agents in all of the extracts, these results suggest a ubiquitous presence of toxicologically active compounds in FCMs made from paper and board, which in itself warrant much greater efforts towards determining both the presence and activities of potential hazardous properties of chemicals used in these products.

Extracts from FCMs have previously been shown to exhibit genotoxicity (Ozaki et al., 2004), ER activity (Vinggaard et al., 2000), and AhR activity (Binderup et al., 2002). In line with this, we observed that two out of the twenty tested FCM extracts were Ames positive, all were AhR active, and around half induced ER activity, suggesting that they are relevant endpoints to include in a broad-based strategy. We also included two other assays related to genotoxicity (p53) and oxidative stress (Nrf2), and found a surprisingly high incidence of activities. Therefore, it may be prudent to include several different assays related to genotoxicity in future test programs.

The fact that all extracts induced AhR activity could suggest that there are natural constituents in paper and board that can act as AhR ligands. Alternatively, paper products may all be contaminated with persistent pollutants that activate this receptor. We previously attempted to determine the compound(s) accounting for the AhR activity in FCM extracts (Bengtström et al., 2016), but unsuccessfully. So the answer as to whether the AhR ligand is endogenous to the base materials used to make paper and board, or constitute ubiquitous

contamination remains unanswered. But in either case, the extent to which the AhR receptor is activated is of concern if the responsible compound(s) proves capable of migrating into foodstuffs.

Estrogenic activity has been reported in both kitchen rolls (Vinggaard et al., 2000) and paper-based take-away food containers (Lopez-Espinosa et al., 2007b), with BPA and selected phthalates detected in 45-100% of the samples (Lopez-Espinosa et al., 2007b; Vinggaard et al., 2000). BPA, DBP, and BBP have previously shown estrogenicity *in vitro* previously (Ghisari and Bonefeld-Jorgensen, 2009; Gould et al., 1998; Kitamura et al., 2005; Krishnan et al., 1993; Mankidy et al., 2013; Paris et al., 2002; Shen et al., 2009; Zhang et al., 2011). This suggests that the occurrences of these compounds are commonplace for paper and board products, and that BPA or phthalates may often be the main drivers of the observed estrogenic effects. In fact, we identified both BPA and the two phthalates DBP and BBP in the pizza box, and our data indicate that these were the compounds responsible for the effect.

The sandwich wrapper (S3) proved to contain AR active compounds. We identified both AA and DHAA in this product, albeit AA in much higher concentrations, and showed that bothwere AR antagonists. AA has previously been reported to inhibit AR activity (Rostkowski et al., 2011) and to inhibit 5α-reductase (Roh et al., 2010), whereas DHAA, to our knowledge, has not been reported to be AR active. Certain FCMs have been reported to contain both DHAA and AA (Ozaki et al., 2006; Weber et al., 2006) which can migrate to food simulants (Ozaki et al., 2006). Therefore, although human exposure data remains insufficient, it appears likely that FCMs constitute a human exposure source of these compounds. Further biomonitoring efforts are required to confirm frequency and concentration levels across different demographics.

The overall strategy is intended to assess inherent hazards posed by FCMs of paper and board in order to facilitate future prioritization efforts. The extraction procedure was therefore developed with no consideration of the intended use of the FCM, which allows for the broadest possible collection on information of the bioactive constituents. Alternative approaches that are based on biological testing on food simulants after migration analyses have been suggested and could potentially be standardized for future test strategies. However, it would be hard to define a single food simulant that can catch all potential problematic chemicals. On the other hand, combining several food simulants is both time and resource demanding. Therefore, to minimize costs - and due to the fact that chemicals in FCM may end up in the environment and then indirectly contaminate foods and recycled FCMs - the use of 'worst case' extractions can be preferable, as it allows for detecting emerging chemicals in FCM. Migration studies can be performed subsequently on the identified chemicals according to standard procedures.

Based on our results from the twenty FCMs made from paper and board, we deem our strategy a valuable approach for identifying emerging chemicals in these products. Further considerations regarding which *in vitro* assays to include and the application of a tiered approach, however, could potentially improve upon the strategy, particularly with regards to time and cost of running large scale screening programs. So too, developing accessible and affordable mass spectral libraries for FCM compounds could greatly enhance the identification process by reducing the number of relevant or required analytical standards for identified peaks. Finally, combining the test protocols with ultrasensitive analytical chemistry would be beneficial, particularly if it targets very potent ligands (such as dioxins) for specific

receptors (such as AhR) in order to rule out if responses have been caused by these ligands, as suggested by (Koster et al., 2014).

5. Conclusion

Various FCMs can contain problematic substances that can become a health issue if they migrate into foods for human consumption. This is especially the case if the consumer is exposed to active substances or other chemicals with similar modes of action from other sources, which can give rise to cocktail effects (Svingen and Vinggaard, 2016). Although current EU regulations clearly state that FCMs cannot contain chemicals that can migrate in amounts that may be hazardous to humans, FCMs in general remains poorly regulated, not least products made from paper and board. To address this, we have developed an effect-directed strategy that can contribute with important knowledge for future risk assessments. By testing different products, we found that the 'contamination level' of certain FCMs can be relatively high, and thus recommend that considerable more efforts be given to these products as potential sources of human exposure to chemicals.

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TABLES

Table 1. *Selected food packaging materials (FCM) of paper and board. The use or intended use of the FCM, the material type, the supplier, the pulp type, and whether printing existed are stated.*

Extract & Usage		Material	Supplier	Pulp type	Printing
S1	Plain paper	Paper	Paper industry	V	No
S2	Baking paper	Paper	Retail	V	No
S3	Sandwich wrapper*	Paper	Retail	V	No
S4	Baking paper	Paper	Retail	V	No
S5	Baking mold	Paper	Retail	V	Yes
S6	Flour bag[a]	Paper	Retail	V	Yes
S7	Pizza box*	Corrugated fiberboard	Retail	R	Yes
S8	Microwave pizza tray	Paperboard	Printing industry	VR	Yes
S9	Susceptor microwave popcorn	Paperboard	Printing industry	VR	Yes
S10	Microwave popcorn bag	Paper	Printing industry	R	Yes
S11	Frozen fish box	Paperboard	Printing industry	VR	Yes
S12	Cereal box[a]	Paperboard	Retail	R	Yes
S13	Cake tray	Paperboard	Printing industry	VR	Yes
S14	Sausage tray	Paperboard	Printing industry	VR	Yes
S15	Tomato punnet	Paperboard	Printing industry	VR	Yes
S16	Imported Chinese 1	Paperboard	Printing industry	R	Yes
S17	Imported Chinese 2	Paperboard	Printing industry	R	Yes
S18	Paperboard, UV print	Paperboard	Printing industry	R	Yes
S19	Paperboard, water-soluble print	Paperboard	Printing industry	R	Yes
S20	Paperboard, offset print	Paperboard	Printing industry	R	Yes

[a]Contained food at purchase, *samples for fractionation, V=virgin pulp/paper, R=recycled paper.

Table 2. *Effect of 20 FCM extracts on activity of the androgen receptor (AR), aryl hydrocarbon receptor (AhR), estrogen receptors (ER), peroxisome-proliferator-activated receptor gamma (PPARγ), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, p53 pathway, and the Ames test.*

Extract & Usage			AR		AhR	ER	PPARγ	Nrf2	P53	Ames
S1	Plain paper	LOEC	0.8	22	22	-	6.5	7.2	-	-
		E_{max}	290	45	115	-	470	286	-	-
S2	Baking paper	LOEC	6.3	0.2	6.3	-	1.9	19	-	-
		E_{max}	73	65	73	-	86	223	-	-
S3	Sandwich wrapper	LOEC	0.8	2.3	21	-	-	7.0	-	-
		E_{max}	532	47	81	-	-	233	-	-
S4	Baking paper	LOEC	-	2.5	7.4	-	-	7.4	-	-
		E_{max}	-	28	224	-	-	234	-	-
S5	Baking mold	LOEC	-	0.7	2.0	-	-	-	-	-
		E_{max}	-	51	174	-	-	-	-	-
S6	Flour bag	LOEC	-	-	0.7	5.9	0.2	5.9	18	-
		E_{max}	-	-	568	82	190	838	154	-
S7	Pizza box	LOEC	-	-	0.005	0.3	-	2.3	2.3	-
		E_{max}	-	-	1040	103	-	776	141	-
S8	Microwave pizza tray	LOEC	0.4	-	0.4	-	-	-	-	POS
		E_{max}	327	-	260	-	-	-	-	
S9	Susceptor microwave popcorn	LOEC	-	-	1.1	-	1.9	-	-	-
		E_{max}	-	-	505	-	97	-	-	-
S10	Microwave popcorn bag	LOEC	-	-	0.04	-	-	-	-	POS
		E_{max}	-	-	229	-	-	-	-	
S11	Frozen fish box	LOEC	-	0.4	0.4	2.1	1.9	1.9	-	-
		E_{max}	-	66	223	63	140	446	-	-
S12	Cereal box	LOEC	-	-	0.04	-	0.7	0.7	-	-
		E_{max}	-	-	329	-	166	877	-	-
S13	Cake tray	LOEC	-	0.5	0.2	-	0.4	4.6	-	-
		E_{max}	-	42	274	-	377	224	-	-
S14	Sausage tray	LOEC	-	-	0.4	2.3	0.6	2.1	2.1	-
		E_{max}	-	-	992	69	60	1645	47	-
S15	Tomato punnet	LOEC	-	-	0.002	0.6	0.2	0.5	-	-
		E_{max}	-	-	1069	105	370	1052	-	-
S16	Imported Chinese 1	LOEC	1.3	-	0.1	1.3	1.1	3.8	11	-
		E_{max}	104	-	670	160	360	1233	124	-
S17	Imported Chinese 2	LOEC	11	11	0.4	3.5	3.1	3.5	-	-
		E_{max}	141	36	701	132	240	292	-	-
S18	Paperboard, UV print	LOEC	-	0.1	0.07	0.1	0.3	0.1	0.4	-
		E_{max}	-	49	634	245	150	828	278	-
S19	Paperboard, water-soluble print	LOEC	-	-	0.09	0.2	-	0.5	4.8	-
		E_{max}	-	-	430	226	-	983	365	-
S20	Paperboard, offset print	LOEC	-	-	0.1	-	-	1.8	-	-
		E_{max}	-	-	869	-	-	1351	-	-

Values are based on data shown in Supplementary Materials. LOEC = Lowest Observed Effect Concentration (cm^2_{FCM}/mL), E_{max} = tentative maximum effects (% change compared to control), green =activation, red = inhibition, white = no effect. POS: positive test. For AR antagonism LOECs were determined as the concentrations at which a ≥ 25% inhibition of AR activation was observed. For AR, AhR, and

ER agonism LOECs were determined as the concentrations causing ≥ 50% activation. LOECs for PPARγ, Nrf2, and P53 were the lowest concentration leading to a statistically significant effect.

Table 3. *Calculated versus measured equivalence factors (EQs) for estrogen receptor agonism of the pizza box and androgen receptor antagonism of the sandwich wrapper. The EQ for identified compounds, bisphenol A (BPA), di-butyl phthalate (DBP), butyl-benzylphthalate (BBP), dehydroabietic acid (DHAA),and abietic acid (AA) were calculated relative to the positive controls,17β-estradiol and hydroxyflutamide for ER and AR activity, respectively.*

Estrogenic activity in pizza box (S7)		
Identified compounds	**Concentration (µM)[a]**	**EEQ (µM)**
BPA	0.08	$1.11*10^{-5}$
DBP	0.19	$1.89*10^{-7}$
BBP	0.07	$1.99*10^{-7}$
Total EEQ_{calc}		$11.5*10^{-6}$
Total EEQ_{meas}		$2.23*10^{-6}$
Antiandrogenic activity in sandwich wrapper (S3)		
Identified compounds	**Concentration (µM)[a]**	**AEQ (µM)**
DHAA	3.9	$2.14*10^{-4}$
AA	485.2	$1.49*10^{-1}$
Total AEQ_{calc}		$14.9*10^{-2}$
Total AEQ_{meas}		$8.84*10^{-2}$

a) Concentrations (µM) in diluted FCM extract at maximum in vitro response

655 **Table 4.** *Total extracted amounts of AR and ER active substances into ethanol from pizza box (S7) and sandwich wrapper (S3) and measured migration into food simulants (Tenax and 50% ethanol) expressed in mg/kg food and in % of the total amount of specific substances present in 6 dm^2 FCM.*

Sample	Substance	Extraction results	Migration into food simulants			Legislative restrictions for plastic FCMs*	
		Total amount of substance in FCM (mg/6 dm^2)	Tenax (mg/kg$_{food}$)	50% ethanol (mg/kg$_{food}$)	Highest migration in % of amount in the sample	Specific migration limit (SML) (mg/kg$_{food}$)	Comments
Sandwich wrapper (S3)	AA	4,5	0.002	0.5	11	60	AA is authorised as a monomer but not as an additive
	DHAA	0,05	n.a.	n.a.	-	-	Not an authorised substance
Pizza box (S7)	BPA	0,13	<0.002		n.a.	0.6	
	DBP	0,37	0.03		8	0.3	DBP is not approved for single-use FCMs
	BBP	0,13	<0.002		n.a.	30	

*Plastics regulation EUC 10/2011, Annex I

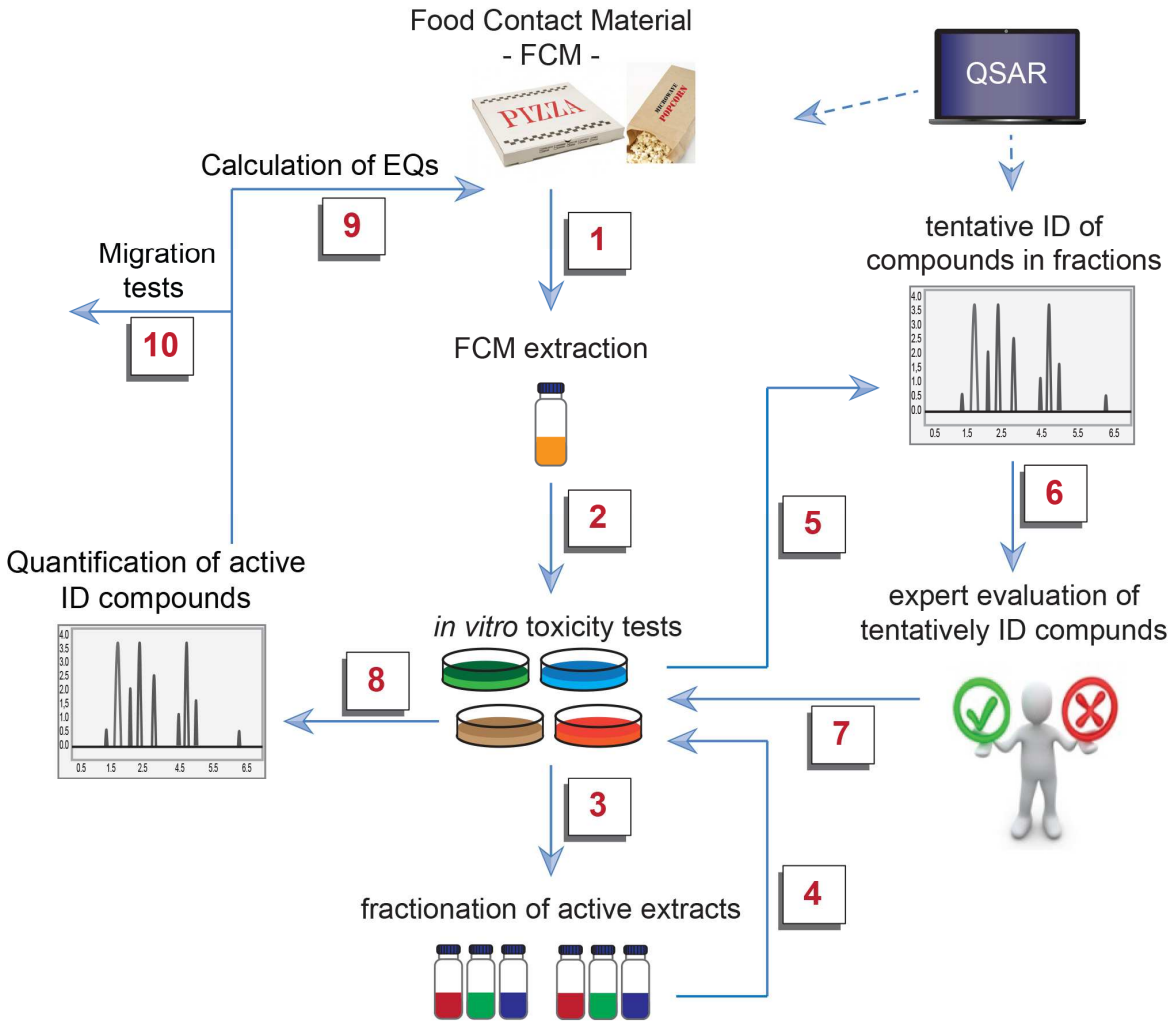
660 SML= Specific Migration Limit; n.a. = could not be determined as levels were below limit of quantification.

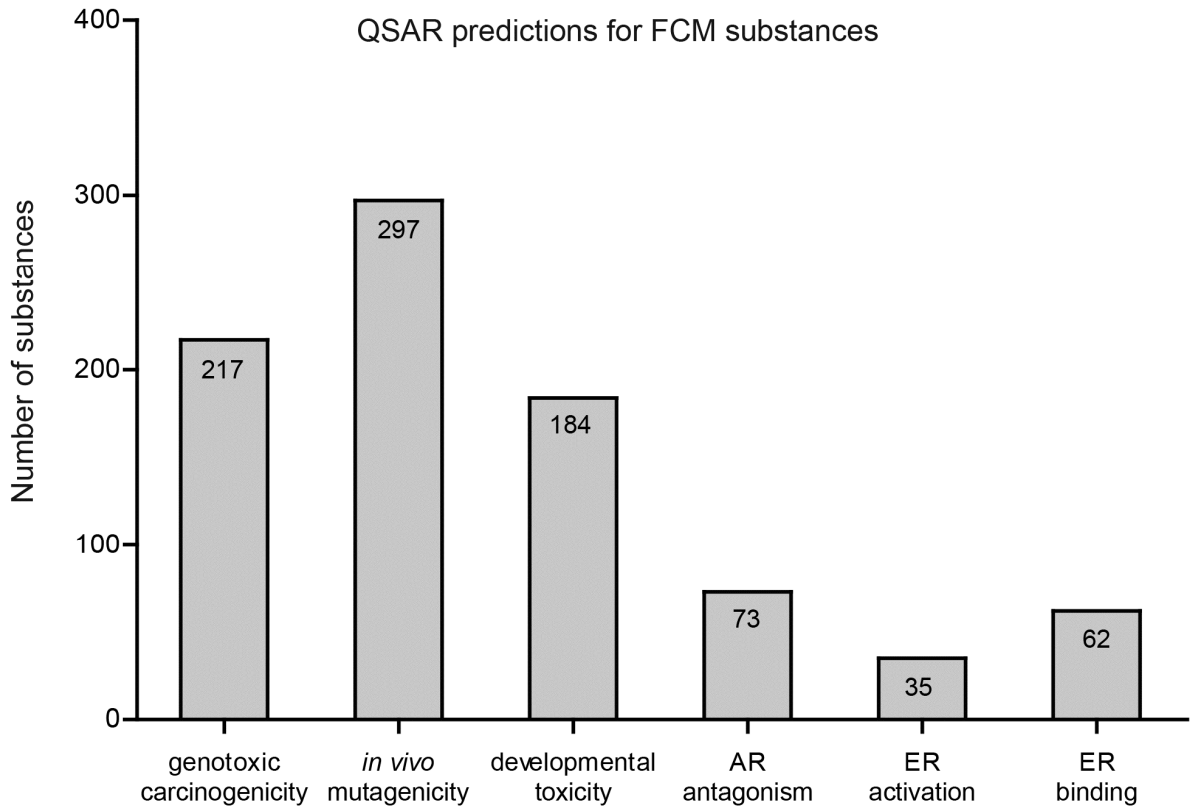
FIGURE LEGENDS

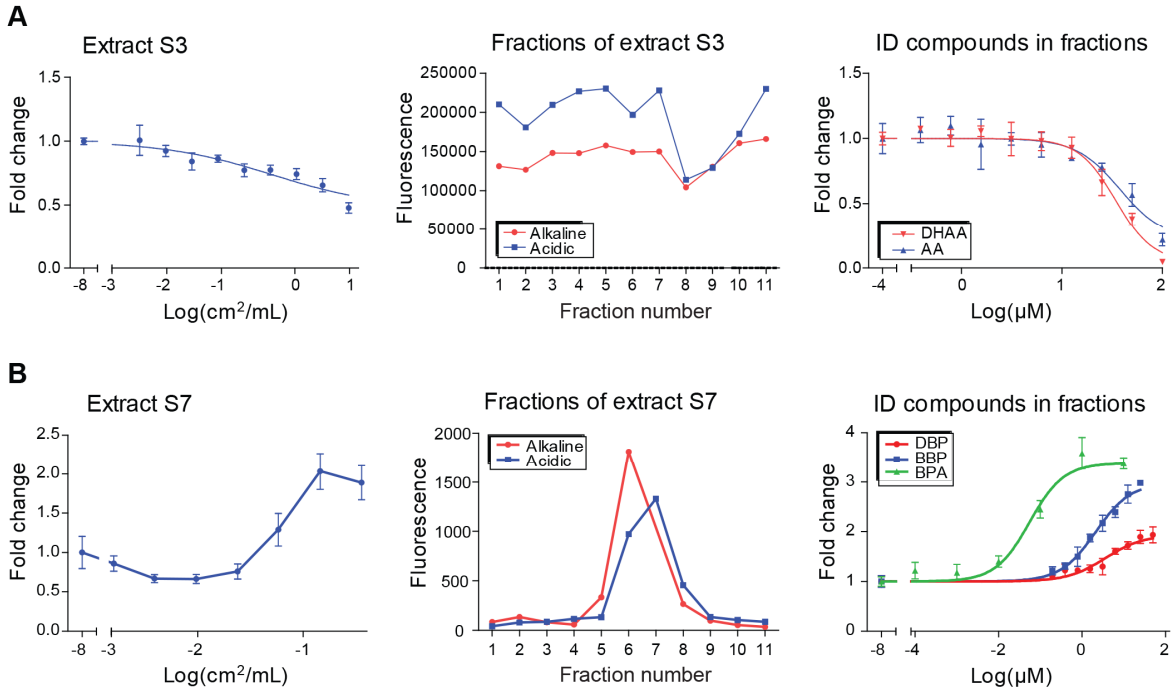
Figure 1: Workflow for the effect-directed strategy for FCMs made from paper and board, 1) preparation of FCM extracts from paper and board, 2) in vitro testing of extracts, 3) fractionation of active extracts, 4) in vitro testing of fractions, 5) tentative compound identification in active fractions, 6) selection of final list of test compounds, 7) in vitro testing of final list compounds, 8) identification and quantification of compounds, 9) calculation of equivalence factors (EQs), 10) migration studies. ID = identification.

Figure 2: QSAR predictions for genotoxic carcinogenicity, *in vivo* mutagenicity, developmental toxicity, *in vitro* estrogen receptor (ER) activation and binding, and androgen receptor (AR) antagonism for 2,076 FCM compounds.

Figure 3: In vitro data from workflow described in Figure 1. Biological activity of FCM extracts (left), fractions of extracts (middle), and identified (ID) biologically active compounds in active fractions and extracts (right). **A)** Androgen receptor (AR) antagonism of sandwich wrapper (S3) extract and fractions as well as compounds therein. **B)** Estrogen receptor (ER) agonism of the pizza box (S7) extract and fractions as well as compounds therein. Graphs are based on one representative experiment of extract, fractions, and ID compounds (means ±SD). BPA data has previously been published in Rosenmai et al. 2014.







Highlights (for review)

- A strategy for an effect-directed analysis of food contact materials has been developed
- Twenty food packaging materials were toxicologically profiled
- Bisphenol A and phthalates were responsible for estrogenic activity of a pizza box
- Abietic acid and dehydroabietic acid were responsible for androgen receptor antagonism in a sandwich wrapper
- A tool for identifying emerging chemicals in food packaging has been developed